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MEMBRANE STATUS OF BOAR SPERMATOZOA AFTER COOLING OR CRYOPRESERVATION

W.M.C Maxwell[1a] and L.A. Johnson[2]

[1]Department of Animal Science, University of Sydney, NSW 2006, Australia
[2]Germplasm and Gamete Physiology Laboratory, Agricultural Research Service, USDA Beltsville, MD 20705, USA

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ABSTRACT

This study tested the hypothesis that sperm membrane changes during cooling contribute substantially to the membrane damage observed after cryopreservation of boar spermatozoa. Flow cytometry was used to assess viability (percentages of live and dead cells) of boar sperm cells after staining with SYBR-14 and propidium iodide (PI) and acrosome status after staining with FITC-pisum sativum agglutenin and PI.

Incubation (38°C, 4 h), cooling (to 15 or 5°C) and freezing reduced the proportion of live spermatozoa compared with those in fresh semen. There were more membrane changes in spermatozoa cooled to 5°C than to 15°C. The proportion of live spermatozoa decreased during processing for cryopreservation and cooling to 5°C, but was unaffected by freezing and thawing if held at 15°C for 3.5 h during cooling. Spermatozoa not held during cooling exhibited further loss of viability after freezing and thawing. Holding the spermatozoa also increased the proportion of acrosome-intact spermatozoa at both 15°C and 5°C and at thawing compared with that of the unheld controls. The results of this study sugget that a substantial proportion of the membrane changes associated with cryopreservation of boar spermatozoa may be attributed to the cooling of the cells to 5°C rather than to the freezing and thawing process, and that sperm membrane changes are reduced when semen is held at 15°C during cooling.

Key words: boar spermatozoa, flow cytometry, cryopreservation, cooling, viability, membrane status

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[a]Correspondence and reprint requests:
Department of Animal Science, University of Sydney, NSW 2006, Australia.
Telephone: +61 2 9351 4864 FAX: +61 2 9351 3957 email: chism@vetsci.su.edu.au

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INTRODUCTION

Damage to the membranes of boar spermatozoa during cryopreservation can reduce fertilizing capacity (10), and necessitate more precise timing of insemination of frozen semen with respect to expected ovulation (15, 17). Watson (19) showed that cooling boar spermatozoa appeared to induce a state equivalent to capacitation based on the proportion of cells displaying the B pattern of chlortetracycline fluorescence, and suggested that these cooling-induced changes may contribute to the reduced fertility of cryopreserved boar spermatozoa. One current method for the cryopreservation of boar semen includes an extended holding period at 15°C as part of the slow cooling to 5°C before freezing (1).

It is important to define the components of the membrane changes that affect sperm function in order to improve semen cryopreservation methods. The objective of this study was to determine which part of the freezing-thawing procedure was most detrimental to membrane integrity of boar spermatozoa: the prefreezing cooling or the freezing-thawing process. The hypothesis was that sperm membrane changes during cooling contribute substantially to membrane damage observed after cryopreservation of boar spermatozoa, and that these changes would be reduced by holding the semen in the diluent during the cooling process. To test this hypothesis, the viability and membrane status of boar spermatozoa were assessed after incubation, cooling and cryopreservation. A preliminary report on this work has been presented elsewhere (11).

MATERIALS AND METHODS

Reagents and Media

All chemicals were of analytical grade. Unless otherwise stated, all media components were purchased from Sigma Chemical Co. (St. Louis, MO), and were made up with Milli-Q® water (Milli-Q UF Plus; Millipore, Bedford MA).

The media used in the experiments consisted of phosphate buffered saline (PBS: 8.5 g NaCL, 1.15 g Na_2HPO_4, 0.23 g NaH_2PO_4), Tyrode's salt solution with or without added bicarbonate (composition of Tyrode's-based media were described prevously; 3); and Beltsville TS (BTS; 9). The PBS and Tyrode's media were buffered with NaOH or HCl to pH 7.4 at room temperature and had a final osmolality of 300 mOsm/kg; they were equilibrated for 30 minutes at 38°C in an atmosphere of 5% carbon dioxide in air before use.

Fluorescent Stains

SYBR-14 and PI. The live cell nucleic acid stain, SYBR-14 (*Ferti*Light(TM) Kit, Molecular Probes, Eugene, OR) was used in combination with propidium iodide (PI) as described by Garner et al. (5). The SYBR-14 was dissolved in anhydrous dimethyl sulfoxide (DMSO; Sigma, D-8779) at a concentration of 1 mg/ml. A working solution of SYBR-14, diluted 1:10 with DMSO, was used for staining (0.1 mg/ml). The PI was dissolved in Tyrode's salt solution with added bicarbonate to make up a working solution of 2 mg/ml.

FITC-PSA and PI. Membrane status of spermatozoa was also assessed after staining with FITC-conguated lectin and PI as described by Graham et al. (6) and Ashworth et al. (3). The FITC-PSA conjugate (Pisum sativum, FITC labelled, salt-free lyophilized powder; Sigma, L-0770) was made up as a working solution of 0.1 mg/ml in PBS without BSA, and the PI was dissolved in Tyrode's salt solution with added bicarbonate to make up a working solution of 2 mg/ml.

Collection and Dilution of Semen

Semen from mature boars (sperm rich fraction) was collected in a thermally protected vessel. The semen was transported to the laboratory and allowed to come to room temperature

(approximately 24°C). Within 30 minutes of collection, semen was extended 1:1 in BTS and assessed for progressive motility and for concentration of spermatozoa using a hemocytometer. Only ejaculates with >70% progressively motile spermatozoa were used.

Incubation and Cooling of Semen

Ten-millilitrre aliquots of extended spermatozoa were sealed in 15-mL conical tubes (Opticul™ polypropylene; Becton Dickinson, Franklin Lakes, NJ). For incubation the tubes were held in a water bath (38°C) for 4 h; for cooling they were placed in a temperature-regulated cabinet and the temperature was reduced at a rate of 0.25°C/min.

Cryopreservation and Thawing of Semen

Semen was processed according to the straw freezing procedure described by Westendorf et al. (20) and Almlid et al. (2) as modified by Almlid and Johnson (1). Briefly, the semen diluted in BTS was placed at 15°C for 3.5 h and centrifuged (800 g, 10 min); the supernatant was then removed, and the spermatozoa were resuspended with lactose-egg yolk extender (LEY; 1) to a concentration of 300 x 10^6 cells/ml. After further cooling to 5°C for 90 min, two parts of LEY-extended semen were mixed with one part LEY + 1.5% Equex STM (Nova Chemical Sales Inc., Scituate, MA) and 6% (v/v) glycerol to give a final freezing concentration of 200 x 10^6 sperm/ml. The diluted and cooled semen was loaded into 0.5-ml straws (IMV, Minneapolis, MN) and placed in liquid nitrogen vapor approximately 3 cm above the level of the liquid nitrogen for 20 min. The straws were then stored in liquid nitrogen. Thawing was achieved by immersing the straws in a circulating waterbath set at 37°C for 20 sec. Immediately after thawing, the semen was processed for assessment as described in the experimental design of the Results section.

Assessment of Membrane Integrity (Viability) and Status of Spermatozoa

Prior to assessment, the spermatozoa were washed twice (centrifugation 200 g, 6 min) with PBS or Tyrodes (see Experimental and Results) and resuspended to 15 x 10^6 spermatozoa per ml.

Motility of spermatozoa by microscopy. Ten µl of resuspended spermatozoa were placed on a microscope slide (37°C), covered with a cover slip, and the percentage progressively motile spermatozoa assessed under phase contrast microscopy (100 x magnification).

Membrane integrity after staining with SYBR-14 and PI. Volumes of 0.27 µl SYBR-14 working solution and 2 µl PI stock solution were added to 500 µl of resuspended spermatozoa using Pipetman P-2 and P-10 (Rainin Instrument Co., Emeryville, CA) and FluoroPel pipette tips (No. T30510F, Ulster Scientific Inc., New Platz, NY). The stained samples were mixed by inversion and incubated in a 36°C heating block for 15 min. The proportions of SYBR-14- (intact membranes, "live") and PI-stained (non-intact membranes, "dead") spermatozoa in each sample were then quantified by flow cytometry (Garner et al. 1994).

Membrane status after staining with FITC-PSA and PI. To 500 µl resuspended spermatozoa 30 µl FITC-PSA and 2 µl PI were added. This suspension was then placed in a 36°C heating block for 15 min. The proportions of FITC-PSA-bound and PI-stained sperm cells were then quantified by flow cytometry.

Flow cytometry. Quantitative data on the spermatozoa fluorescently stained with SYBR-14/PI and with FITC-PSA/PI were collected using an EPICS PROFILE II (Coulter Corporation, Inc.,

Miami, FL). This flow cytometer, which has an air-cooled Argon laser (488 nm), was equipped with the PowerPak option, which provides three-color fluorescence detection in addition to side and forward light scatter parameters. The side and forward light scatter parameters were gated so that only those cells possessing the light scatter characteristics of spermatozoa were analyzed for fluorescence. A total of 5,000 (SYBR-14/PI) or 10,000 (FITC-PSA/PI) spermatozoa were analyzed for the log of their fluorescence for each sample. Green fluorescence 1 (LFL 1: SYBR-14 or FITC) was collected through a 525-nm bandpass filter, while red fluorescence parameters (PI), fluorescence 2 (LFL 2) and fluorescence 3 (LFL 3) were collected through 575-nm band and 635-nm bandpass filters, respectively. Compensation (25%: SYBR-14/PI; 40%: FITC-PSA/PI) was used to minimize spillover of green fluorescence into the 635-nm red channel (LFL 3), and to make cytogram patterns easier to evaluate. Single-parameter histograms for LFL1 and LFL 3 were acquired along with two-parameter cytograms of LFL 1 and LFL 3).

Histogram analyses. The histograms were analyzed using the Coulter Histogram Analysis Program (Coulter Corporation, Miami, FL). The relative distributions of the green- and red-stained sperm populations were collected as dotplot cytograms for LFL1 and LFL 3. The particular populations of the cytograms were isolated using gated window analysis and quantified based on the methods described earlier (5, 14). For SYBR-14/PI-stained spermatozoa, the predominant red-stained (PI), green-stained (SYBR-14) and dual-stained spermatozoa were quantified. For FITC-PSA/PI-stained spermatozoa, detector settings were standardized using unsorted spermatozoa that had been stained with FITC-PSA alone and PI alone. Windows were set to delineate the subpopulation categories as described by Maxwell et al. (14): 1) spermatozoa stained with PI regardless of the FITC signal (cells with nonintact membranes); 2) spermatozoa unstained with PI and showing a lower FITC fluorescence signal ("low-fluorescence live" or "acrosome intact" cells); and 3) spermatozoa unstained with PI and showing a higher FITC fluorescence signal ("high-fluorescence live" or "acrosome reacted" cells).

Statistical Analyses

Each experiment was replicated three times. The data were first analyzed by Harvey's least squares maximum likelihood analysis of variance (Harvey 1987). The least squares means and standard deviations presented in the tables and figures are those for untransformed percentages. For statistical analyses, however, percentage values were subjected to arcsin transformation before applying unpaired Students t-tests to means and standard deviations for each datum point (Snedecor and Cochran 1980).

RESULTS

Experiment 1

We used a 2 x 4 x 4 factorial design to assess the effect of medium and semen treatment on membrane integrity without cryopreservation. The following factors were examined: 1) resuspension medium: PBS vs Tyrode's + bicarbonate; 2) type of spermatozoa: fresh vs incubated vs cooled to 15°C vs cooled to 5°C; and 3) boars: sperm rich fractions of ejaculates from 4 boars.

The spermatozoa were washed and resuspended with PBS or Tyrode's + bicarbonate before incubation, cooling or fresh assessment. After treatment, the resuspended spermatozoa were divided into 2 aliquots for staining with SYBR-14/PI and FITC-PSA/PI for assessment of viability and acrosome status, respectively.

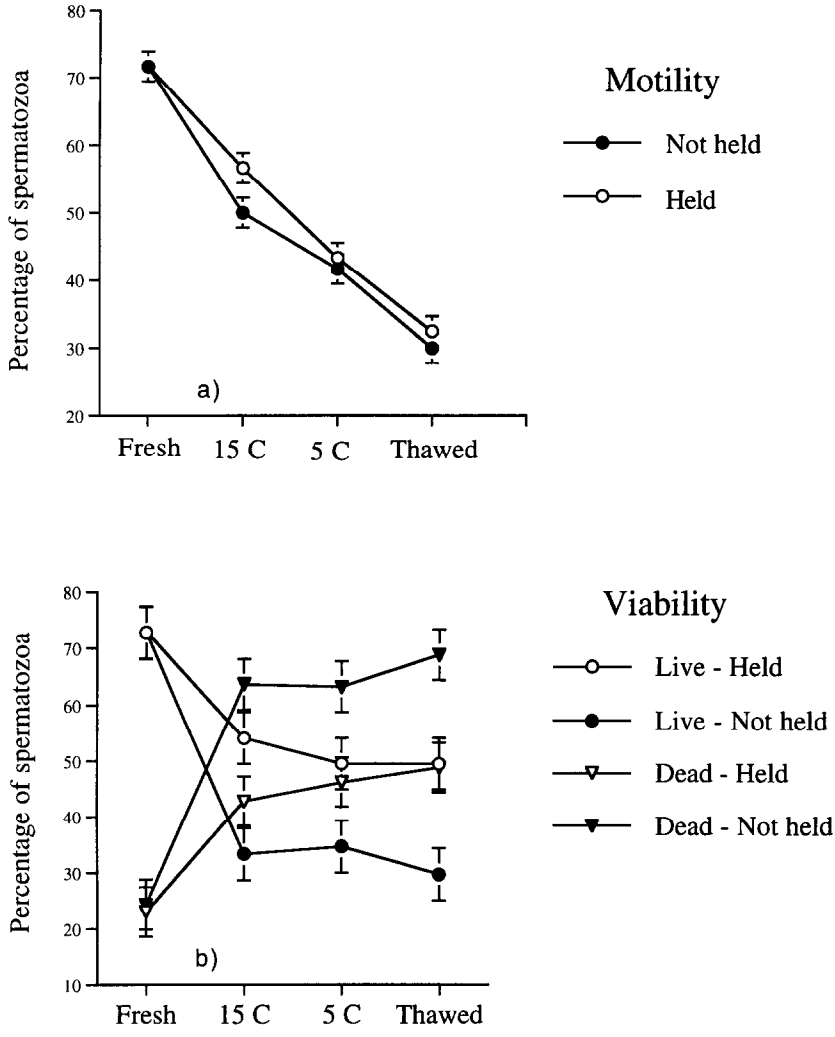
Considerable variability between boars was observed in the percentage of live, dead and acrosome reacted spermatozoa (Table 1). This variation was reflected in first order interactions

Table 1. Effect of medium for resuspension of spermatozoa (Medium), type of spermatozoa and boars on percentage of live[a], dead[a], dual-stained[a] spermatozoa and on the proportion of live spermatozoa with low (acrosome intact) and high FITC fluorescence (acrosome reacted)[b]. Data are least-squares means.

Treatment	% spermatozoa				
	Live[a]	Dead[a]	Dual[a]	Intact[b]	Reacted[b]
Medium					
PBS	59.1	38.6	1.9	38.9	40.8
Tyrode's	63.1	32.7	2.8	34.8	49.7
SEM	*2.98*	*2.90*	*0.32*	*3.22*	*2.54*
Probability	*NS*	*NS*	*NS*	*NS*	*<0.05*
Type of spermatozoa					
Fresh	74.4	22.9	2.0	44.2	32.3
Incubated	62.4	34.2	2.5	40.5	46.7
15°C	70.8	25.7	2.1	42.4	43.6
5°C	36.7	59.8	2.6	20.2	58.3
SEM	*4.21*	*4.10*	*0.45*	*4.55*	*3.59*
Probability	*<0.001*	*<0.001*	*NS*	*<0.05*	*<0.01*
Boar ID no.					
0043	73.0	23.1	3.0	47.3	23.5
2948	56.7	40.0	2.7	38.2	51.9
0704	46.2	52.2	1.2	35.3	56.9
1043	68.4	27.4	2.3	26.5	48.6
SEM	*4.21*	*4.10*	*0.45*	*4.55*	*3.59*
Probability	*<0.01*	*<0.01*	*NS*	*NS*	*<0.001*

[a]Determined by fluorescent staining with SYBR-14 and propidium iodide and flow cytometry.

[b]Determined by fluorescent staining with FITC-PSA and propidium iodide and flow cytometry (data excludes PI-positive cells)


Percentage of spermatozoa
80
70
60
50
40
30
20
a)
Fresh
15 C
5 C
Thawed
Motility
Not held
Held
Percentage of spermatozoa
80
70
60
50
40
30
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10
b)
Fresh
15 C
5 C
Thawed
Viability
Live - Held
Live - Not held
Dead - Held
Dead - Not held

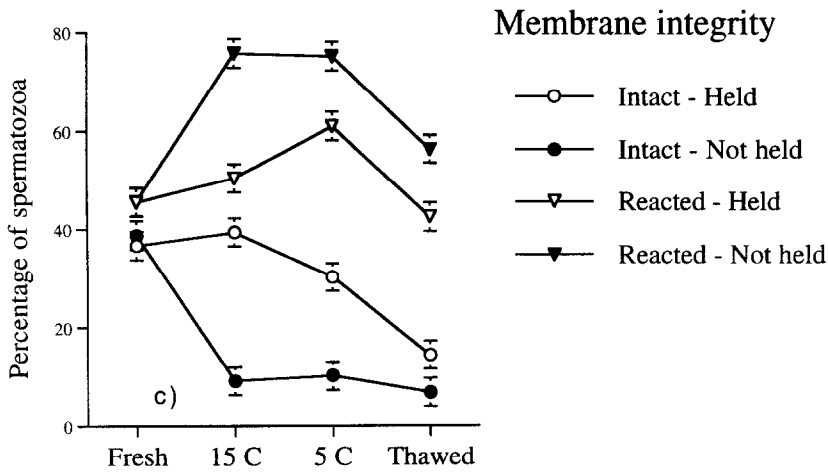

Figure 1. Effect of holding time at 15°C for 3.5 hours during cooling (Held vs Not held) and stage of cryopreservation at which spermatozoa were assessed (Fresh: after dilution in BTS, before cooling; 15°C: after cooling to 15°C, centrifugation and resuspension in lactose-yolk; 5°C: after cooling to 5°C, before addition of glycerol and freezing; Thawed: after thawing and removal of glycerol by centrifugation) on (a) motility, (b) viability (percentage of live and dead spermatozoa after staining with SYBR-14/PI), and c) membrane integrity of spermatozoa (percentage with low [intact] and high fluorescence [reacted acrosomes] after staining with FITC-PSA/PI). Motility was assessed by phase contrast microscopy, and viability and membrane integrity by flow cytometry. Data are least squares means + SEM and are pooled for boars and resuspension media.

between boars and medium for resuspension of spermatozoa on the proportions of spermatozoa with high fluorescence (acrosome reacted) after staining with FITC-PSA/PI. More spermatozoa were acrosome-reacted in Tyrode's than in PBS ($P<0.05$, Table 1) medium, except for Boars 0043 and 0704, in which the medium had no effect on the percentage of acrosome-reacted cells.

The proportions of live and dead sperm cells after staining with SYBR-14/PI and of acrosome-intact cells after staining with FITC-PSA/PI were unaffected by the resuspension medium, but more spermatozoa were live ($P<0.001$) and acrosome intact ($P<0.05$), and fewer were dead ($P<0.001$) in fresh, incubated and cooled (15°C) semen than in semen cooled to 5°C (Table 1).

Experiment 2

A 2 x 4 x 2 x 3 factorial was used to examine the effect of holding time at 15°C on motility, viability and membrane integrity of spermatozoa at processing for cryopreservation and after thawing. The following factors were examined: 1) medium for resuspension of spermatozoa: Tyrode's + bicarbonate vs Tyrode's - bicarbonate; 2) stage of cryopreservation: fresh vs 15°C vs 5°C vs thawed; 3) holding at 15°C: held vs not held; 4) boars: sperm rich fractions from 3 boars.

Spermatozoa were washed and resuspended in Tyrode's medium with or without bicarbonate before assessment fresh (after dilution with BTS), at 15°C (after cooling to 15°C, centrifugation, resuspension with lactose-yolk and holding), at 5°C (after cooling to 5°C but before addition of glycerol and freezing), and thawed (after thawing and centrifugation to remove glycerol). After resuspension, the spermatozoa were divided into 3 aliquots for assessment of motility by microscopy, viability by flow cytometry after staining with SYBR-14/PI, and membrane status by flow cytometry after staining with FITC-PSA/PI.

The motility of spermatozoa declined as processing for cryopreservation progressed ($P<0.001$), but motility was unaffected by holding at 15°C for 3,5 h (Figure 1). The proportion of live spermatozoa also decreased during processing and cooling to 5°C ($P<0.001$) but was unaffected by freezing and thawing when held at 15°C for 3.5 h during cooling. Spermatozoa not held during cooling exhibited further loss of viability after thawing compared with the assessment at 5°C before freezing ($P<0.001$; Figure 1). Compared with spermatozoa not held at 15°C during cooling, holding spermatozoa increased the proportion of acrosome intact at both 15°C and 5°C and at thawing. The proportion of acrosome-intact cells remained relatively constant during cooling when semen was held at 15°C but declined substantialy when not held ($P<0.001$; Figure 1).

More spermatozoa were live (41.7 vs 34.9±2.30%) and fewer were acrosome-reacted (54.4 vs 58.7±1.45%) when resuspended without than with bicarbonate in Tyrode's medium ($P<0.05$).

DISCUSSION

Changes in sperm membrane integrity were identified in this study using FITC-PSA, which is reported to bind specifically to acrosomal contents (5). Graham et al. (6) showed that fluorescently labelled PSA could be used in flow cytometry to assess the percentage of cells with or without intact acrosomes; this was based on comparisons with napthol yellow/erythrosin B staining combined with an assessment of the percentage of cells with intact acrosomes when the acrosome reaction was induced with LPC. We adopted a standard description of FITC-PSA stained spermatozoa similar to that used by Ashworth et al. (3). Cells with low FITC fluorescence were described as acrosome-intact, while those with high FITC fluorescence were designated acrosome-reacted. However, it should be noted that a proportion of spermatozoa in each population showed varying intensities of fluorescence, which may have reflected gradual rather than specific changes in membrane destabilization. To detect changes taking place specifically in the live sperm

population, we also stained the spermatozoa with PI, and the PI-positive cells were excluded from the estimate of acrosome-intact (low) and reacted cells (high FITC fluorescence). However, the PI-positive sperm population was not homogeneous, with so-called dead cells often clearly divided into 2 subpopulations with low and high FITC fluorescence. These subpopulations may indicate a gradual change in membrane status associated with the death of acrosome-reacted spermatozoa. Membrane changes measured during flow cytometry may also reflect additional damage incurred by the spermatozoa as a result of dilution and passage through the flow cytometer (14).

The key role of bicarbonate in the process of capacitation has been described in detail by Harrison (7). An attempt was made in this study to inhibit and enable capacitation-like membrane changes in spermatozoa subjected to incubation, cooling and cryopreservation by resuspending the spermatozoa in media free of bicarbonate or with added bicarbonate, respectively. As expected, compared with a medium without bicarbonate (PBS), Tyrode's with added bicarbonate enabled more membrane change during incubation and cooling of boar spermatozoa, as assessed by FITC-PSA/PI fluorescence; but it did not influence sperm viability as assessed by SYBR-14/PI staining (Experiment 1). However, in Experiment 2, bicarbonate, when added after processing and shortly before assessment of spermatozoa, elicited a change in both the acrosome status and viability of spermatozoa. Ashworth et al. (3), also using FITC-congugated lectins, found that bicarbonate specifically enabled slow membrane changes akin to the unmasking of underlying glycoconjugates, that could bind to the lectins.

The hypothesis was tested in this study that much of the membrane destabiliszation associated with cryopreservation might be attributable to the process of cooling spermatozoa prior to freezing. The latter hypothesis was supported by the data of Watson (19) on CTC fluorescence patterns in fresh boar spermatozoa and after cooling the cells to 15 or 5°C. Watson showed that cooling appeared to induce a state equivalent to capacitation, based on the proportion of spermatozoa displaying the B pattern of CTC fluorescence. The results of Experiment 1 are essentially equivalent to those of Watson (19). However, one current method for the cryopreservation of boar semen includes an extended holding time at 15°C as part of the slow cooling procedure to 5°C (1). This holding period has been reported to confer a form of de-capacitation to the sperm cell, as the procedure is notably conducted in the presence of seminal plasma. The practical use of cryopreserved boar semen, which is dependent on insemination close to the time of ovulation (15), lends support to the hypothesis that the frozen-thawed spermatozoa may be functionally capacitated (13).

In Experiment 2, our finding like that of Watson (19) showed the importance of cooling in the destabilization of sperm membranes. We extended this observation to the cryopreservation process used for boar spermatozoa. The cryopreservation process (1) used an initial dilution in a noncapacitating medium (BTS), followed by either no holding or a 3.5 h holding period at 15°C. The cooling of semen in BTS medium caused less membrane destabilization than cooling spermatozoa to 15 or 5°C in the presence of capacitating medium used in Experiment 1, as assessed by fluorescence after staining with FITC-PSA/PI, when the semen was held at 15°C. Most of the membrane destabilization assessed after staining with SYBR-14/PI was caused by the cooling process rather than by freezing and thawing (Figure 1). This finding suggests that it is the reduction in temperature during cooling, rather than ice crystal formation during freeze-thawing or the toxic effects of glycerol, that is responsible for most of the gross membrane damage to cryopreserved spermatozoa.

It is well established that boar spermatozoa are particularly susceptible to cold shock when cooled below 15°C. If, on the other hand, the semen is held at room temperature in seminal plasma for several hours the spermatozoa acquire resistance to cold shock (16). This knowledge is utilized in freezing protocols for boar spermatozoa such as the one used in this study, where it is recommended that semen be held for 3 to 4 h after dilution at approximately 15°C and usually (1)

but not necessarily (12) before the addition of glycerol. It is not clear what changes take place in the cells during the holding period, but Watson (19) suggests that itinvolves neither alterations in the protein configuration of the plasma membrane nor variation in carbohydrate terminals of the surface glycocalyx. Rather, it may be related to the lipid composition of the bilayer affecting the fluidity of the plasma membrane but not the fertilizing capacity of the cells. Whatever the mechanism, it is clear that a period of holding the semen at 15°C can inhibit and even partially reverse membrane destabilization. The latter is evident in the difference between held and not held spermatozoa when assessed at 15°C for viability and membrane status (Figure 1).

In conclusion, a substantial proportion of the membrane changes associated with cryopreservation of boar spermatozoa may be attributed to the cooling of the cells, rather than the freezing and thawing process *per se*, and there is less membrane change if the semen is held at 15°C during cooling. Further tests are required to determine the mechanism of changes in the membrane status of spermatozoa following cryopreservation, and to determine whether fertility is similarly affected by cooling rather than freeze-thawing.

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